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ARTICLE

Characterization and Pathogenicity of *Pseudopestalotiopsis vietnamensis* Causing Gray Blight of Wuyi Rock Tea (*Camellia sinensis*) in China and Specific Mechanisms of Disease Infection

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ABSTRACT

Gray blight disease (GBD) causes significant losses in tea production in China. Although genes and biological processes involved in resistance to fungal disease in tea plants have been identified, specific mechanisms of the GBD infection process remain unknown. In this study, morphological and multi-gene (*TEF-TUB-ITS*) phylogenetic characteristics were used to identify isolate CLBB1 of *Pseudopestalotiopsis vietnamensis*. Pathogenicity tests confirmed that isolate CLBB1 from tea leaves caused GBD in the susceptible tea cultivar Wuyi Rock (*Camellia sinensis* var. *sinensis* cv. Shuixian). Spores began to germinate 24 h after infection (hai), and after 48 h, elongated fungal hyphae formed from a single conidium. Transcriptome analysis revealed that 482, 517, and 369 genes were differentially expressed at 24, 48, and 72 hai, respectively, in Wuyi Rock tea leaves. Isolate CLBB1 infection elicited phenotype-related responses and activated defense-related pathways, including plant–pathogen interaction, MAPK signaling, and plant hormone signal transduction, suggesting a possible mechanism underlying phenotype-based susceptibility to CLBB1. Thus, a new *Ps. vietnamensis* strain causing GBD in the tea cultivar 'Shuixian' was discovered in this study. Transcriptome analysis indicated that pathogen invasion activated chitin-related MAPK pathways and that tea plants required a hormone to restrict CLBB1.

KEYWORDS

Chitin-related MAPK pathways; gray blight disease; *Pseudopestalotiopsis*; tea; transcriptional analysis

1 Introduction

Tea, coffee, and cocoa are the three most popular nonalcoholic beverages worldwide, with tea being especially popular in Asia [1]. Tea contains a variety of secondary metabolites and aromas, such as polyphenols, polysaccharides, and γ-amino butyric acids, which may reduce the risk of diabetes, cardiovascular disease, Parkinson's disease, and Alzheimer's disease [2–4]. Polyphenols in tea can also help prevent a few types of cancer [5,6]. In addition, drinking tea can reduce mental stress and anxiety [7]. Tea is mainly produced from young leaves and buds of tea plants (*Camellia sinensis* (L.) O. Kuntze), and it is a valuable commercial crop in China, India, Sri Lanka, Vietnam, Indonesia, and Myanmar [8]. To improve tea quality, an effective approach is to select varieties of tea plants with good traits. Currently, tea is commercially cultivated in over 50 countries, including China, which has hundreds of tea germplasm resources from which to select tea cultivars [9]. In China, tea is further classified into different types on the basis of growing conditions, harvesting procedures, and processing methods, and types include black, green, yellow, white, oolong, and Pu-erh teas [10]. Oolong tea is a partially fermented tea with the fragrance of green tea and the smoothness of black tea, in addition to a distinct woody fragrance. Oolong tea has high levels of theanine, polyphenols, polysaccharides, and total catechins [10–12]. The anti-obesity components in oolong tea may reduce the risk of type II diabetes [13–15].

Because of the distinct flavor and excellent health benefits, oolong tea is popular among most Chinese. Traditional oolong tea is primarily grown in Fujian Province in southeastern China. Wuyi Rock tea is a well-known type of oolong tea and one of China's top ten tea types [16]. Named after its natural habitat, Wuyi Rock tea grows from cracked rocks of Wuyi Mountain. The two main cultivars of Wuyi Rock tea in production are 'Rougui' and 'Shuixian' [12,16]. 'Shuixian' (*C. sinensis* var. *sinensis* cv. Shuixian) is an autotriploid ($2n = 3x = 45$) variety that was bred using asexual reproduction to maintain stable traits across generations. The variety was designated as a "national variety" in 1985.

Pathogenic microorganisms are a threat to tea plants, and warm and humid conditions promote the occurrence of most tea plant diseases. Pathogenic microorganisms such as anthracnose (*Colletotrichum*) typically cause lesions and disease spots on tea leaves [17]. Despite numerous studies on interactions between pathogens and tea plants, the dominant pathogens causing significant losses of Wuyi Rock tea remain unknown. Gray blight disease (GBD) is a common disease that causes significant yield losses in a variety of economically important crops, including apple, blueberry, coconut, grape, guava, hazelnut, lychee, mango, ginger, and tea [18]. It is also one of the most common tea diseases in China, with outbreaks in Jiangsu, Fujian, Anhui, Sichuan, Guizhou, Hunan, Yunnan, Zhejiang, and Guangdong [19]. Lesions are the most common symptom of GBD. At the beginning of infection, small yellow or green spots appear on tea leaves at leaf tips or margins that gradually develop into brown semicircular, circular, or irregular shapes and become grayish white as lesions become more visible [18].

Pestalotiopsis-like species can cause necrotic lesions on *C. sinensis* [20]. Gray blight lesion symptoms are also observed during the growing season of Wuyi Rock tea (*C. sinensis* var. *sinensis* cv. Shuixian) in Wuyi Mountain, causing yield losses. Furthermore, because 'Shuixian' is autotriploid, it is more susceptible to pathogens. However, the pathogen responsible for GBD in Wuyi Mountain has not yet been identified and characterized. The aim of this study was to identify the isolates causing GBD in Wuyi Rock tea cultivar 'Shuixian' based on morphological characteristics and multigene phylogenetic analysis, and reveal mechanisms of disease infection using RNA sequencing (RNA-seq) and reverse-transcription quantitative PCR.

2 Materials and Methods

2.1 Plant Materials, Fungal Isolates, and Treatments

The study was conducted at the Tea Garden of Wuyi University in Wuyishan, Fujian Province, China (27°72'67"N, 118°00'47"E; 130 m above sea level). Twenty tea leaves with disease symptoms were

collected in June 2018. Leaves were surface-sterilized and incubated on potato dextrose agar (PDA, 200 g potato + 2% [w/v] dextrose + 2% [w/v] agar) for fungal strains. Petri dishes were incubated at 25°C ± 2°C under light of 3,000 to 4,000 lux until fungal colonies were observed. Pure cultures were obtained from hyphal tips collected at margins of colonies. In pathogenicity tests, mechanical inoculation of plants was performed *in vitro* based on methods described by Wang et al. [20]. Three-year-old tea plants (*C. sinensis* var. *sinensis* cv. Shuixian) were used as experimental materials. The third and fourth healthy mature leaves were sampled at 1, 2, 3, 4, and 10 days after inoculation (dai). Alcoholic trypan blue and 3, 3-diamino benzidine hydrochloride (DAB) staining assays were performed, and fungal structures were examined under a Leica DM2500 microscope (Germany) [21]. To observe the ultrastructure of CLBB1, gluta fixative (4%) was used to observe hyphae and conidia with a scanning electron microscope (TESCAN VEGA3, Czech).

2.2 DNA Extraction, PCR Amplification, and Sequencing

From mycelia grown on PDA plates, DNA was extracted with a PlantGen DNA Kit of CW Biotech (Beijing, China). The genes internal transcribed spacer (*ITS*) of rDNA, translation elongation factor−1α (*TEF*), and β-tubulin (*TUB*) were amplified and sequenced with primers ITS1/ITS4, EF-1α-F/R, and β-tub-F/R, respectively [22]. Primer sequences are listed in Table 1. The 2xTaq PCR Mix of LabLead were used for PCR amplifications (Fuzhou, China), and Sangon Biotech Co., Ltd. (Shanghai, China) conducted the sequencing.

Table 1: Primer pairs used in the study

Genes	Primer sequence (5'–3')
ITS1	TCC GTA GGT GAA CCT GCG G
ITS4	TCC TCC GCT TAT TGA TAT GC
EF-1α-F	AGT GCG GTG GTA TAG ACA AG
EF-1α-R	TTG CCC TCC TTC TTG ACA AG
β-tub-F	CGA AGT TGT CAG GGC GGA AA
β-tub-R	CTA CGA CGA CCT TGA ACG CT
RT-CsCHITINASE III-F	GTT TAC TTA ACT GGC GCG
RT-CsCHITINASE III-R	CTT ACT CCA AAG CAT CAC
RT-CsMPK3-F	TTG GTC GTG GTG CTT ATG
RT-CsMPK3-R	CCG TAG TGG TGG AGG AAT
RT-CsPR-F	AAC CTT GCT AAG GGC
RT-CsPR-R	CTA ATA AGG ACG TTG
RT-CsPR4-F	AGC GCT GCT AAT GTT AGG
RT-CsPR4-R	GCC CTT TCC ATC AGT GTC
RT-CsWIN1-L1-F	GTA ATG TCG TCG GTG TTG
RT-CsWIN1-L1-R	CTC TTT TCC CAA CCC ATC
RT-Cs18S-F	CCT GAG AAA CGG CTA CCA CA
RT-Cs18S-R	CAC CAG ACT TGC CCT CCA

2.3 Phylogenetic Analysis

Sequences of *ITS*, *TEF*, and *TUB* loci connected end to end were analyzed to investigate the phylogenetic relationships among *Pseudopestalotiopsis* and *Neopestalotiopsis* species using DNA sequences available from the GenBank (Table 2). DNASTAR (Lasergene 7.0) was used to obtain consensus sequences from sequences generated from ITS, EF-1α and β-tub forward and reverse primers. Combination sequence data obtained from the three gene regions were aligned using ClustalX (http://www.clustal.org/). Before phylogenetic analysis, ambiguous sequences at the start and the end were deleted, and gaps were manually adjusted to optimize the alignment. Maximum parsimony (MP) and maximum likelihood (ML) were conducted with branch-and-bound and heuristic searches as implemented in MEGA7.0 with the default options method. Clade stability was evaluated in a bootstrap analysis with 1,000 replicates, random sequence additions with maximum trees set at 10,000, and other default parameters in MEGA.

Table 2: Strains and NCBI GenBank accession numbers used in this study

Species	Isolate	Location	Host	GenBank accession number		
				ITS	TUB	TEF
Pseudopestalotiopsis vietnamensis	CLBB1	Fujian, China	*Camellia sinensis*	MK909901	-	-
Pseudopestalotiopsis vietnamensis	3-KW-2016–17	Hue, Vietnam	Unknown	LC114037	LC114057	LC114077
Pseudopestalotiopsis myanmarina	1-KW-2016–12	Hue, Vietnam	*Prunus* sp.	LC114032	LC114052	LC114072
Pseudopestalotiopsis. myanmarina	1-KW-2016–13	Hue, Vietnam	*Cinnamomum zeylanicum*	LC114033	LC114053	LC114073
Pseudopestalotiopsis sp.	2-KW-2016–10	Kawthaung, Myanmar	*Ixora* sp.	LC114030	LC114050	LC114070
Pseudopestalotiopsis sp.	2-KW-2016-11	Kawthaung, Myanmar	*Kandelia obovata*	LC114031	LC114051	LC114071
Pseudopestalotiopsis sp.	4-KW-2016-16	Ho Chi Minh, Vietnam	Unknown	LC114036	LC114056	LC114076
Pseudopestalotiopsis sp.	4-KW-2016-19	Hanoi, Vietnam	Unknown	LC114039	LC114059	LC114079
Pseudopestalotiopsis camellia-sinensis	LC3010	Fujian, China	*Camellia sinensis*	KX894936	KX895268	KX895153
Pseudopestalotiopsis camelliae	LC3487	Guangxi, China	*Camellia sinensis*	KX894984	KX895315	KX895201
Pseudopestalotiopsis camelliae	LC3490	Guangxi, China	*Camellia sinensis*	KX894985	KX895316	KX895202
Neopestalotiopsis sp.	SPugra1	Colombia	*Psidium guajava*	KR493579	KR493674	KR493650
Neopestalotiopsis sp.	BTca2	Colombia	*Psidium guajava*	KR493535	KR493738	KR493602
Neopestalotiopsis sp.	SVpa7	Colombia	*Psidium guajava*	KR493585	KR493693	KR493656
Neopestalotiopsis sp.	SVpo6	Colombia	*Psidium guajava*	KR493565	KR493701	KR493637
Neopestalotiopsis asiatica	MFLUCC12-0286	Hunan, China	Unknown	JX398983	JX399018	JX399049
Neopestalotiopsis umbrinospora	MFLUCC12-0285	Guangxi, China	Unknown	JX398984	JX399019	JX399050
Seiridium camelliae	SD096	China	*Camellia reticulata*	JQ683725	JQ683709	JQ683741

2.4 RNA Isolation, Library Construction, and Sequencing

To construct RNA-seq libraries, CLBB1 was cultured on PDA and incubated at 25°C ± 2°C under light of 3,000 to 4,000 lux for 10 days to promote sporulation. Conidia were harvested and suspended in sterile distilled water. Conidia concentration was determined using a hemocytometer and adjusted to 1×10^6 mL^{-1} for inoculation. The third and fourth healthy mature leaves of three-year-old tea plants were wounded on the upper surface with a sterile needle, and 20 µL of conidial suspension was applied to the wound. An equal volume of sterile water was applied as a mock inoculation. Inoculated and mock leaf samples were collected from tea plants at 0, 24, 48, and 72 h after inoculation (hai). Samples were immediately frozen in liquid nitrogen and then stored at −80°C until processed further. Three biological replicates were used in each experiment.

2.5 Library Preparation for Transcriptome Sequencing

To generate sequencing libraries, RNA of each leaf sample was extracted using a NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's protocol and guidelines. Concentration of RNA was measured on a Qubit2.0 Fluorometer, and RNA was diluted to 1.5 ng/µL for RNA sequencing. Library quality was assessed on an Agilent Bioanalyzer 2100 System. The Novogene Corporation (Beijing, China) performed library construction and RNA-seq, according to the manufacturer's protocols. A total of 21 libraries (with three biological replicates per sample) were sequenced on an Illumina Novaseq platform, and 150-bp paired-end reads were generated.

2.6 Quality Control and Reads Mapping with the Reference Genome

Raw reads in fastq format were filtered using in-house perl scripts. Filtering primarily included removing reads containing an adapter or poly-N and those of low quality from raw data. To obtain high-quality clean reads, Q20 and Q30 values and GC contents were calculated before downstream analyses to ensure accuracy. The reference genome of 'Shuchazao' and gene model annotations were downloaded from the Tea Plant Information Archive database (http://tpia.teaplant.org/) [23–25], and paired-end reads were mapped to the tea plant reference genome sequence using Hisat2 v2.0.5 [26].

2.7 Quantification of Gene Expression Levels and Differential Expression Analysis

Fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM) was used to determine gene expression levels. FeatureCounts v1.5.0-p3 was used to count numbers of reads mapped to each gene, following FPKM calculation for each gene based on its length. To compare differences in expression patterns among groups (with biological replications), the DESeq2 R package (1.16.1) was used [27]. Resulting p-values were adjusted using Benjamin and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted p-value < 0.05 found by DESeq2 were assigned as differentially expressed.

2.8 GO and KEGG Enrichment Analyses of Differentially Expressed Genes

Gene ontology (GO) [28] and enrichment analysis and the statistical enrichment of differentially expressed genes in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were performed using the clusterProfiler R package.

2.9 Reverse-Transcription Quantitative PCR Verification

To verify results of RNA-seq data, leaf samples of H_2O and CLBB1-inoculated Wuyi Rock tea 'Shuixian', 'Rougui', and 'Shuchazao' cultivars were collected at 0, 24, 48, and 72 hai. Total RNA was extracted with an RNAprep Pure Kit (Tiangen, China). First-strand cDNA from 2 µg of total RNA was synthesized using a NovoScript® Plus All-in-one 1st Strand cDNA Synthesis SuperMix Kit (NovoProtein, China). Accumulation of transcripts was examined using a NovoStart® SYBR qPCR

SuperMix Plus Kit (NovoProtein) with a Bio-rad CFX96 (California, USA). Each reaction system contained 10 μL of SYBR Premix, 7.2 μL of ddH_2O, 2 μL of diluted cDNA, and 0.4 μL of forward/reverse primer. Reverse-transcription quantitative PCR (RT-qPCR) conditions consisted of a preliminary step at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s, and an extension step at 72°C for 30 s. Tea 18S rRNA was used to normalize transcript abundance. All gene-specific primers (RT-) are listed in Table 1 (*CsCHITINASE*, *CsMPK3*, *CsPR*, *CsPR4*, *CsWIN1-L1*, and *Cs18S*). Three biological replicates were used in each experiment. Statistical analysis of RT-qPCR data was performed with DPS7.05.

3 Results

3.1 Identification of CLBB1, A New Isolate of a Pestalotiopsis-Like Genus

To identify the microorganism species causing GBD of Wuyi Rock tea in Fujian Province, fungal strain CLBB1 was screened and purified from leaves of Wuyi Rock tea (*C. sinensis* var. *sinensis* cv. Shuixian) exhibiting typical GBD symptoms in the Tea Garden of Wuyi University at Wuyi Mountain. Harvested diseased, susceptible leaves had large round lesions with dark brown discoloration at leaf edges.

Colony diameter was 52 mm at 3 dai under daylight at 25°C on PDA (Fig. 1A), and over the next three days, the colony spread covering large portions of the plate and produced conidia under aerial mycelium of raised curves (Fig. 1B). There were obvious dark dots on curves with abundant conidia on the plate at 9 dai (Fig. 1C). From 6 dai, observed from the bottom, the culture appeared yellowish white, with gradual accumulation of brown, most likely because CLBB1 produced pale brown pigments released into the medium (Figs. 1D–1F). Germination and developmental course of spores and hyphae in PDA culture were consistent. The white aerial mycelium produced many branches with smooth surfaces at 3 dai (Figs. 1G–1I). Conidia had the following characteristics: five cells, fusiform to ellipsoid, straight to slightly curved, four-septate, 22.4 to 31.7 μm × 5.6 to 7.3 μm (mean = 27.4 μm × 6.2 μm, n = 30), basal cell conic or obconic, hyaline, thin and smooth-walled, 5.5 to 7.1 μm long (mean = 6.5 μm, n = 30) slightly constricted at septa, three pale brown or olivaceous concolorous median cells, and three hyaline apical appendages arising from the apex (Figs. 1J–1L).

3.2 Pathogenicity of CLBB1

Although *Pestalotiopsis*-like species on tea have been reported [18,19], the infection process on tea plants has not been described. In this study, three-year-old Wuyi Rock tea (*C. sinensis* var. *sinensis* cv. Shuixian) plants were infected with CLBB1 spores and examined for disease responses at 10 dai. Sterile water was used as the control. Leaves inoculated with CLBB1 had small brown necrotic spots on front and back sides at 10 dai. Symptoms were similar to those occurring in the field. There were no changes in sterile water-infected sites (Figs. 2A and 2B). Koch's postulates were tested by re-isolation and morphological comparison.

To examine the early stage of fungal development on tea leaves, infected leaves at various times were stained with trypan blue to observe fungal structures. CLBB1 spore germination and penetration pegs at infection sites were observed in epidermal cells at 24 hai (Fig. 2C). At 48 hai, elongated fungal hyphae formed from a single conidium (Fig. 2D). At 72 hai, CLBB1 spores developed extensive branched hyphae with secondary germ tubes that invaded underlying epidermal cells (Figs. 2E and 2F). Infected leaves were also stained with 3, 3-DAB, and there were few accumulations of H_2O_2 in infection sites at 72 hai (Fig. 2E).

3.3 Phylogenetic Tree Analysis

The CLBB1 *ITS* sequence (MK909901) analyzed by BLASTn showed that most isolates of the pathogen had 99% sequence homology with strains of *Pestalotiopsis*-like species in the GenBank. To

further analyze the specific species of *Pseudopestalotiopsis* and *Neopestalotiopsis*, phylogenetic tree analyses were conducted derived from the combined *ITS*, partial *β-tubulin* (*TUB*), and partial *translation elongation factor 1α* (*TEF*) gene regions. Sequence data of CLBB1 and reference strains of *Pestalotiopsis*-like species were obtained from the GenBank (Table 2).

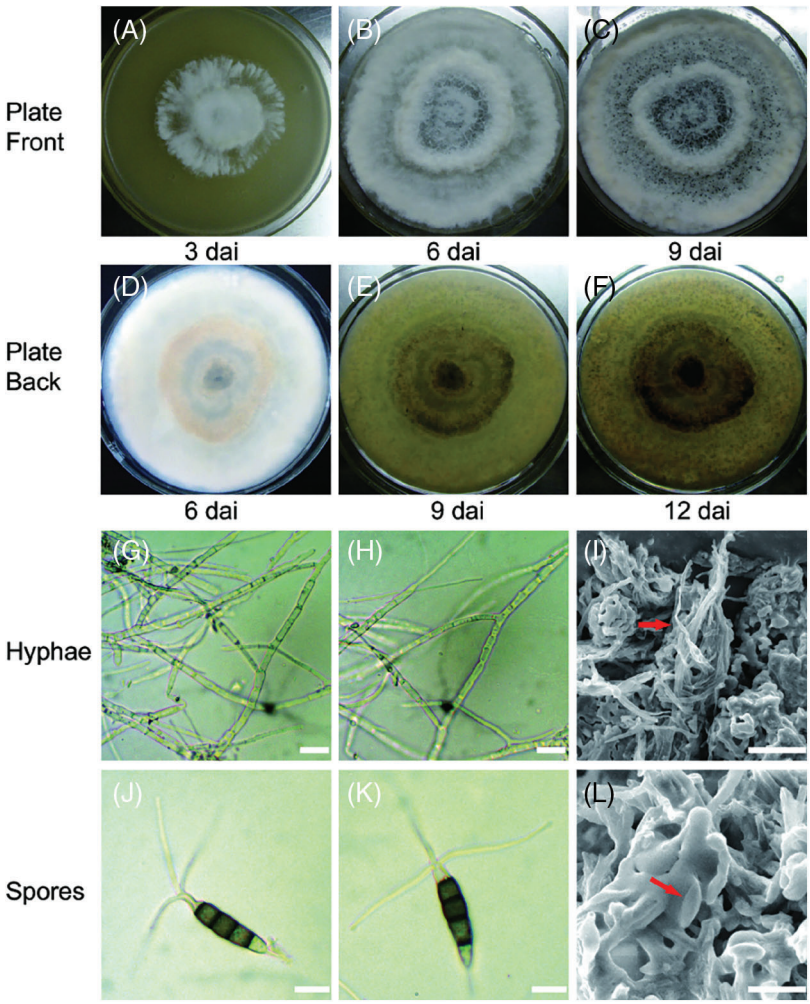

Figure 1: Culture traits and morphology of CLBB1 isolated from symptomatic tea leaves and grown on 90 mm PDA plates. (A–F) Representative culture traits of CLBB1 on PDA at 25°C at 3, 6, 9, and 12 days after inoculation (dai). (G–I) Images of hyphae of CLBB1. Bar = 25 µm. (J–L) Conidial morphology of CLBB1. Bar = 10 µm

From the varied lengths of sequences obtained from NCBI, regions containing many leading or trailing gaps were removed from the *ITS*, *TUB*, and *TEF* alignment. Sequences contained 500 nucleotides including gaps for *ITS*, 340 bp for *TUB*, and 905 bp for *TEF*. Phylogenetic trees were constructed using the MP method. Tree topology of the best tree revealed by ML analyses was identical to that of the MP tree (not shown). Species were separated into two clades including the genera *Neopestalotiopsis* and *Pseudopestalotiopsis* with high bootstrap support. Isolate CLBB1 was in the clade of *Pseudopestalotiopsis* (Fig. 3) and was most closely related to *Ps. vietnamensis* isolate 3-KW-2016–17 collected in Vietnam [29]. Thus, CLBB1 was

considered as *Ps. vietnamensis* on the basis of morphological characteristics and multigene phylogenetic analyses of *ITS*, *TUB*, and *TEF*.

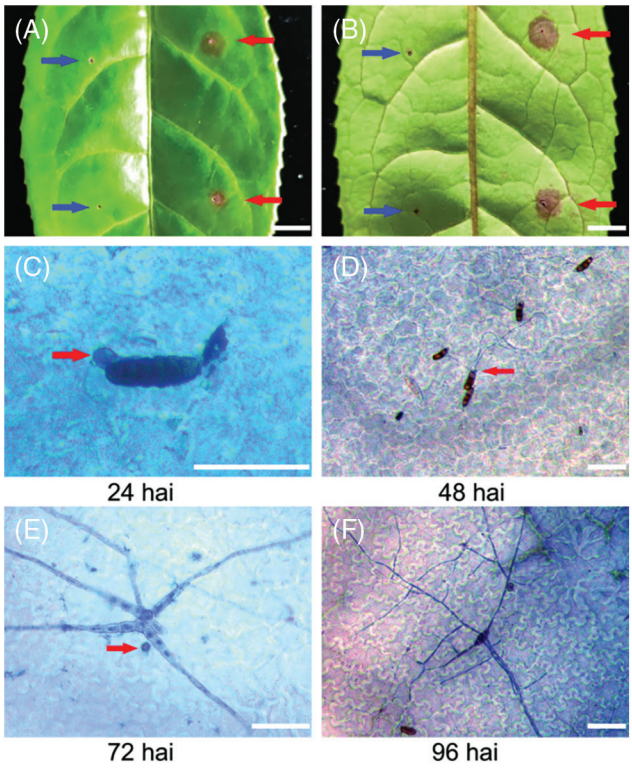

Figure 2: Infection progress of CLBB1 on host tea leaves at 24, 48, 72, and 96 h after infection (hai). (A, B) representative symptoms on leaves removed from three-year-old 'Shuixian' tea plants inoculated with CLBB1 at 10 days after inoculation. Red and blue arrows indicate CLBB1-infected and water-treated sites, respectively. Bar = 0.5 cm. (C–F) Trypan blue staining of representative leaves inoculated with CLBB1 at various times (hai). Red arrows indicate (C) penetration peg, (D) elongated fungal hyphae, and (E) H_2O_2 accumulation in infection site. Bar = 25 μm

3.4 CLBB1 Induced Extensive Immune Responses in Tea Plants

To further investigate the general immune response of tea against CLBB1 at different times after inoculation, RNA was extracted, and 21 sequencing libraries were generated from water (as the control) and CLBB1-infected samples at three different times, with three biological replicates per sample. In total, 145.92 G of high-quality clean data were produced by paired-end Illumina RNA-seq, and each library produced over 40,000,000 clean bases with an average of 97.42% at Q20 and a minimum of 91.72% at Q30. The percentage of clean reads in each library mapped either to a unique location or to multiple genomic locations was greater than 88.4% (Table 3).

Genes were globally differentially expressed, and more than 4,000 differentially expressed genes (DEGs) were identified in each sample. To reduce the deviation caused by negative factors such as environment, buffer, and wound by punch inoculation, sterile water was dropped on wound sites of tea leaves as an inoculation control. The DEGs found in pathogen-inoculated samples but not expressed in water-treated or uninoculated samples were analyzed at 24, 48, and 72 hai. As shown in the Venn diagram (Fig. 4A), 428, 517, and 369 genes were detected at 24, 48, and 72 hai, respectively. The largest number of DEGS at 48 hai was consistent with hyphal formation of CLBB1 fungi at 48 hai. Notably,

only one DEG was expressed at all three times after inoculation, whereas 35 DEGs were co-expressed at 24 and 48 hai and also at 48 and 72 hai. In addition, 13 DEGs were co-expressed between 24 and 72 hai. Therefore, data suggested that CLBB1 activated expression of various genes at certain times after inoculation to increase pathogenicity or induce host defense.

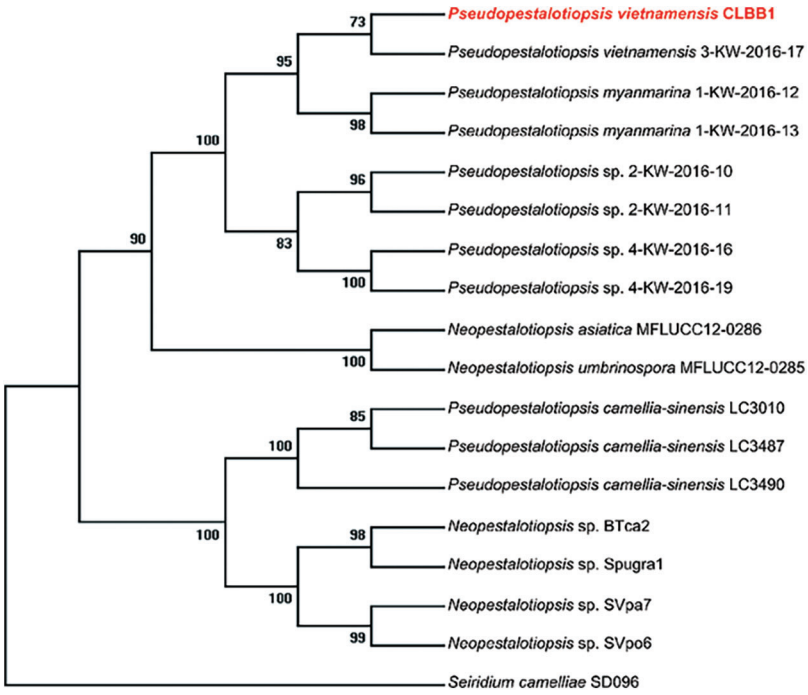

Figure 3: Molecular phylogenetic tree of *Ps. vietnamensis* isolate CLBB1 based on maximum parsimony generated from combined (*TEF–TUB–ITS*) alignment. The tree was rooted to *Seiridium camelliae* SD096. *Ps. vietnamensis* strain CLBB1 was in red and bold

Table 3: Summary of read mapping for RNA-sequencing

Sample	Raw_Reads	Clean_Reads	Clean_Bases	Q20	Q30	GC content	Total_Map
CK_1	50353210	49967776	7.5G	97.26	92.3	44.16	44290088 (88.64%)
CK_2	49146702	48013952	7.2G	97.17	92.18	43.91	42814667 (89.17%)
CK_3	46549688	45457602	6.82G	97.18	92.26	43.77	40390091 (88.85%)
water_24_1	42835296	41632246	6.24G	97.06	91.98	43.71	36802940 (88.4%)
water_24_2	42327030	40962158	6.14G	97.21	92.28	43.81	36377703 (88.81%)
water_24_3	59943460	58924486	8.84G	97.56	92.98	43.45	51909391 (88.09%)
CLBB1_24_1	42340176	40875080	6.13G	97.32	92.52	43.92	36462592 (89.2%)
CLBB1_24_2	47673436	46347522	6.95G	97.35	92.61	43.78	40965372 (88.39%)
CLBB1_24_3	48305248	46914506	7.04G	97.46	92.82	43.59	41781782 (89.06%)
water_48_1	48787260	48256262	7.24G	97.75	93.44	43.33	42770208 (88.63%)
water_48_2	49037448	47583246	7.14G	97.37	92.58	43.64	42939852 (90.24%)

(Continued)

Table 3 (continued)

Sample	Raw_Reads	Clean_Reads	Clean_Bases	Q20	Q30	GC content	Total_Map
water_48_3	48028828	46830766	7.02G	97.51	92.88	43.86	42179289 (90.07%)
CLBB1_48_1	48173436	46717400	7.01G	96.96	91.72	43.88	41447091 (88.72%)
CLBB1_48_2	53898472	52202632	7.83G	97.38	92.64	43.97	46788791 (89.63%)
CLBB1_48_3	51735026	50216676	7.53G	97.22	92.29	43.67	44558987 (88.73%)
water_72_1	40989824	39572566	5.94G	97.47	92.87	44.03	35431029 (89.53%)
water_72_2	43993082	42651694	6.4G	97.33	92.52	43.74	37987771 (89.07%)
water_72_3	44774064	43336140	6.5G	97.55	93.01	44.17	38924317 (89.82%)
CLBB1_72_1	46140200	44545610	6.68G	97.6	93.07	43.48	39468252 (88.6%)
CLBB1_72_2	48860252	47068532	7.06G	97.8	93.55	44.12	41615108 (88.41%)
CLBB1_72_3	46646254	44746112	6.71G	97.67	93.26	43.76	39883093 (89.13%)

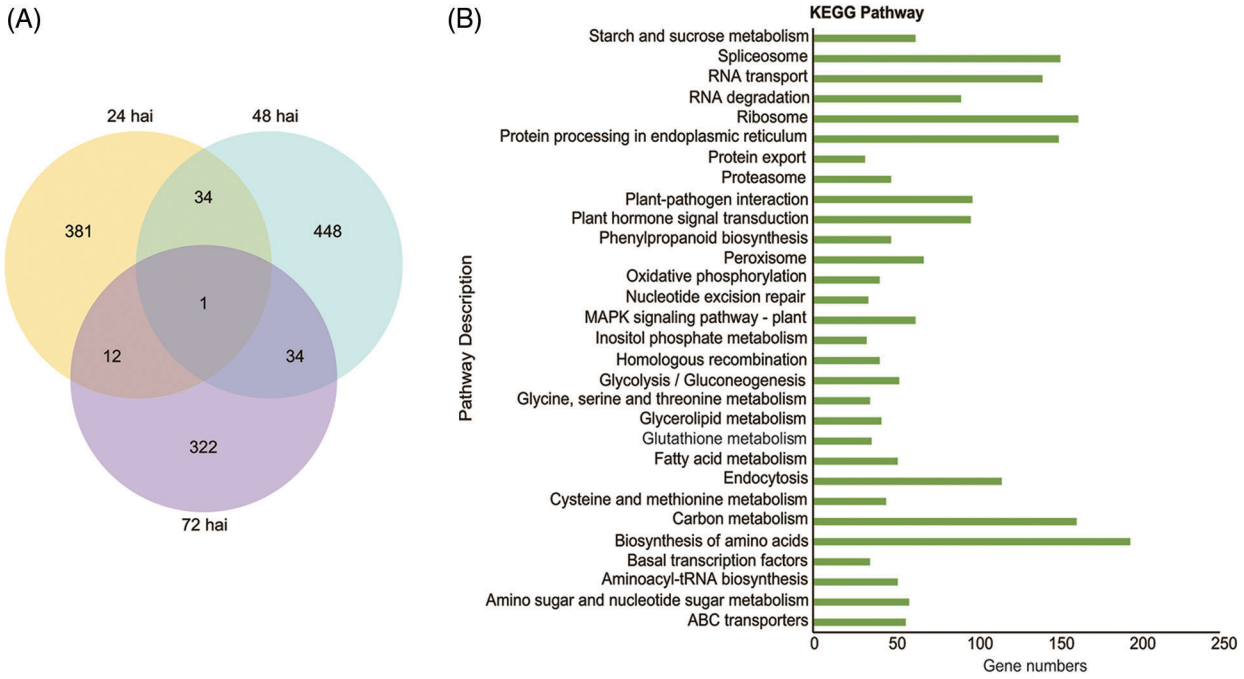

Figure 4: Transcripts induced by CLBB1 at three times after inoculation of tea leaves. (A) Venn diagram of numbers of differentially expressed genes (DEGs) with CLBB1 infection at 24, 48, and 72 h after inoculation (hai). (B) Significantly enriched KEGG pathways of DEGs after CLBB1 inoculation

Gene functions in higher-ordered pathways were investigated using KEGG analysis. The DEGs were highly enriched in RNA and protein biosynthesis, transportation, processing, and modification pathways, such as “spliceosome”, “RNA transport”, “ribosome”, “biosynthesis of amino acids”, and “protein processing in endoplasmic reticulum”. The enriched pathways indicated tea plants synthesized proteins in defense against CLBB1. Transcriptome data showed DEGs were also enriched in “plant-pathogen interaction”, “Mitogen Activated Protein Kinase (MAPK) signaling”, and “plant hormone signal transduction” pathways after CLBB1 infection (Fig. 4B). In addition, the endocytosis pathway was also

enriched, which is involved in the immune response in *Arabidopsis thaliana* [21]. Collectively, the results revealed that CLBB1 induced extensive defense responses in tea plants, particularly by gene enrichment in protein kinase MAPK and plant resistance hormone transduction pathways.

According to GO analysis, many genes in tea plants were activated in resisting pathogen invasion at different stages. A total of 1,232 DEGs were classified into three GO categories, i.e., biological process, molecular function, and cellular component. Most DEGs involved in metabolic processes were enriched at the three times after inoculation (Fig. 5). Genes involved in chitin catabolic and metabolic processes and chitin-responsive (chitin binding, chitinase activity) genes were activated at 24 hai (Fig. 5A). In addition, many sequence-specific DNA-binding genes were expressed, which might induce early defense response gene expression against CLBB1 invasion (Fig. 5B). Genes responsive to pathogens and stress were highly expressed at 48 and 72 hai (Figs. 5B and 5C), which was consistent with growth of the pathogen.

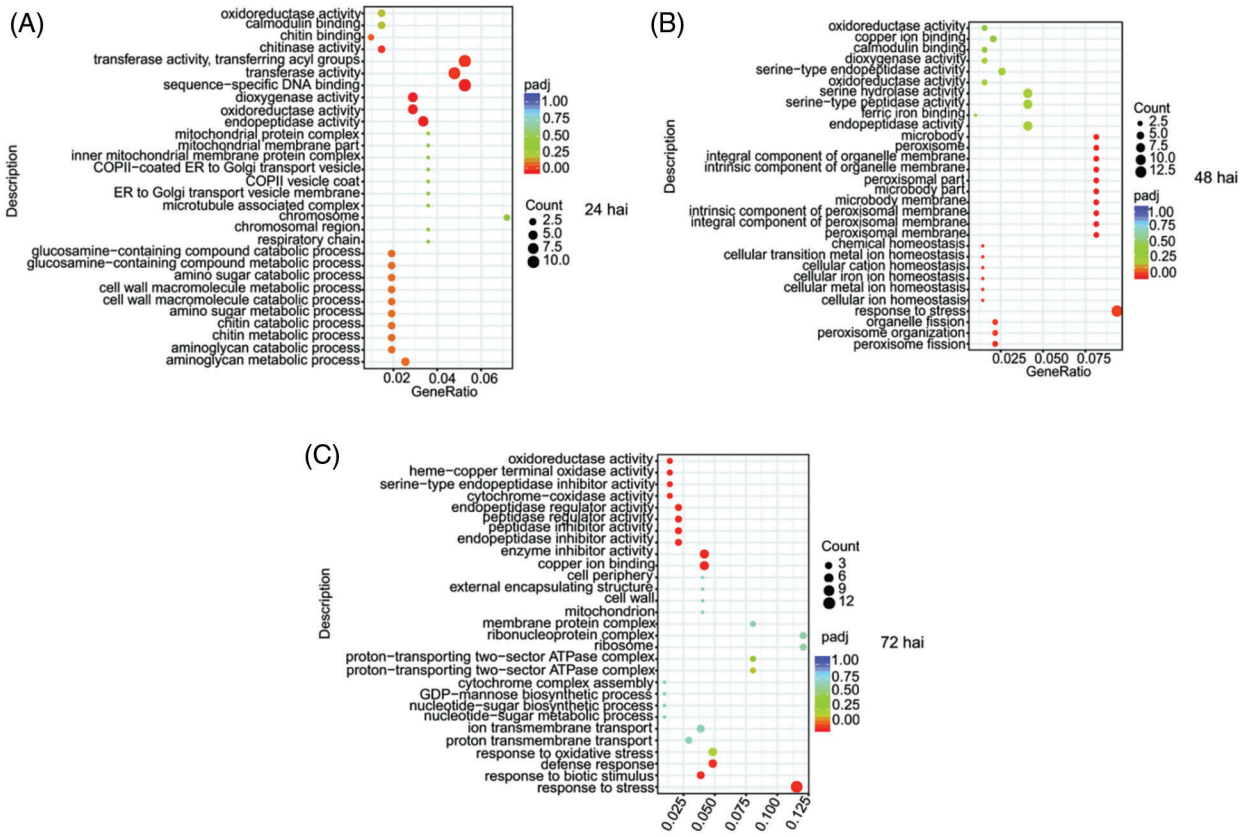

Figure 5: Gene ontology enrichment analysis of differentially expressed genes induced by CLBB1 at three times after inoculation (hai, hours after inoculation). (A) 24 hai, (B) 48 hai, (C) 72 hai

3.5 Reverse-Transcription Quantitative PCR Verification of Candidate Differentially Expressed Genes

To verify genes enriched in "chitin catabolic", "MAPK signaling", and "plant hormone signal transduction" pathways of plant immunity, RT-qPCR was performed to determine expression patterns of five candidate DEGs. Chitinases isolated from plants can limit the growth of chitin-containing fungi, and overexpressed chitinases in plants can resist infection of different fungal pathogens [30]. After CLBB1 infection, the expression level of *CsCHITINASE III* (*TEA002526*) was highly up-regulated at 48 hai in tea cultivar 'Shuixian' (Fig. 6A). To investigate expression levels of DEGs in other cultivars,

transcripts of DEGs were also examined in commercial varieties ‘Shuchazao’ and ‘Rougui’. ‘Shuchazao’ represented *Camellia sinensis* var. *sinensis*, and its whole genome was sequenced. In addition to ‘Shuixian’, ‘Rougui’ is also a Wuyi Rock tea. With CLBB1 infection in ‘Shuchazao’, accumulation of *CsCHITINASE III* transcripts was highest at 24 hai. However, at 72 hai, *CsCHITINASE III* transcript expression levels in CLBB1-inoculated ‘Rougui’ plants were significantly higher than those in the control.

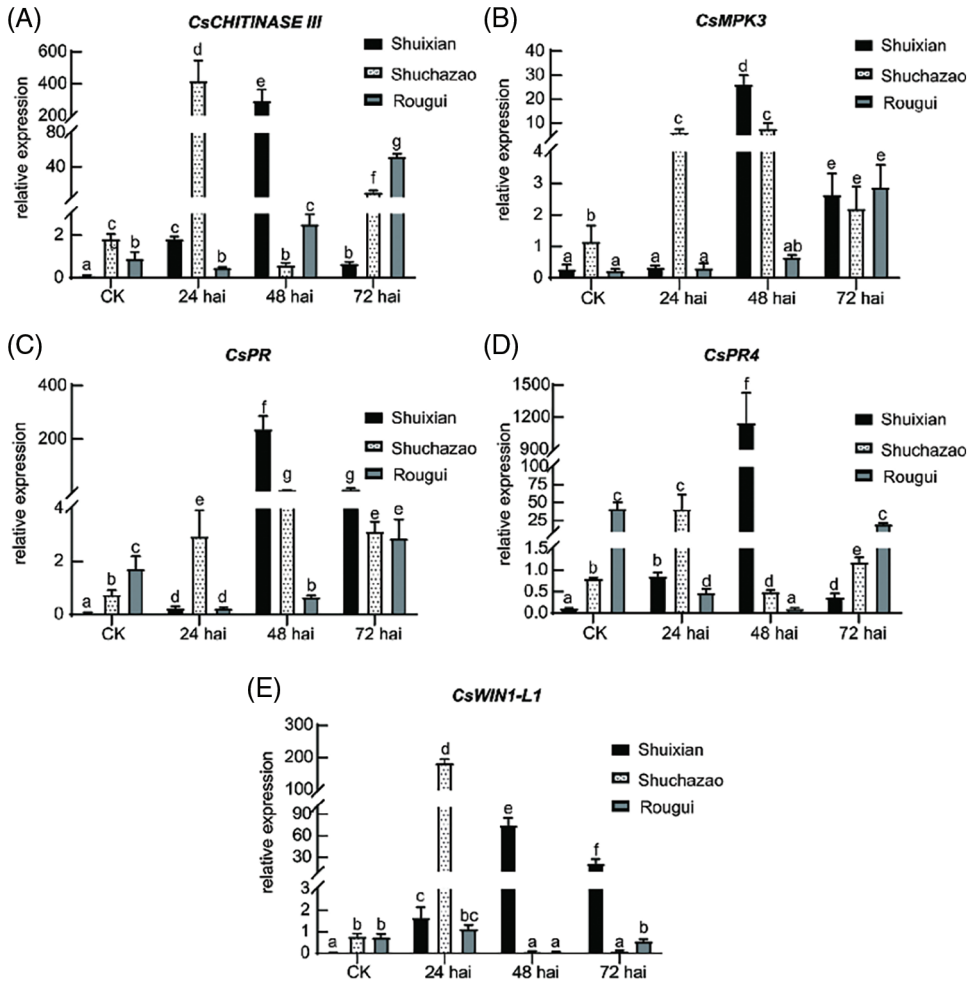

Figure 6: Relative expression levels of defense-related genes in ‘Shuixian’, ‘Shuchazao’, and ‘Rougui’ tea cultivars. Transcript accumulation of defense-related genes was examined by reverse-transcription quantitative PCR, with *Cs18S* used as the internal control. Relative transcriptional levels are shown for (A) *CsCHITINASE III*, (B) *CsMPK3*, (C) *CsPR*, (D) *CsPR4*, and (E) *CsWIN1-L1*. CK represents the uninoculated relative expression amount at 0 hai. Values are the mean and standard deviation obtained from three independent biological samples. Three technical replicates for each biological sample were examined in the experiment. Lowercase letters indicate significant differences ($p < 0.05$; one-way ANOVA). Experiments were repeated three times with similar results

After pathogen infection, plants activate extensive responses to pathogens. In particular, activation of *MPKs* and high expression of pathogenesis-related genes are typical responses against pathogen invasion in PAMP-triggered immunity (PTI) [31–33]. In this study, *CsMPK3* (*TEA026040*) expression level was highly elevated by CLBB1 at 24, 48, and 72 hai in ‘Shuchazao’, ‘Shuixian’, and ‘Rougui’, respectively

(Fig. 6B), which was consistent with expression levels of *CsCHITINASE III* (Fig. 6A). Transcript levels of *CsPR* (*TEA004541*) and *CsPR4* (*TEA013240*) were also measured from 0 to 72 hai with CLBB1 infection. Before inoculation, *CsPR* and *CsPR4* transcript levels were very low in all varieties, but basal expression levels of *CsPR* and *CsPR4* were higher in 'Rougui' than in 'Shuixian' and 'Shuchazao'. With CLBB1 infection, *CsPR* and *CsPR4* transcripts accumulated at the highest levels at 48 hai in 'Shuixian' (Figs. 6C and 6D).

Plants biosynthesize plant resistance hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) to defend against pathogens [32]. To determine whether defense-related hormones were induced by CLBB1, expression level of APETALA2/ethylene-responsive transcription factor *CsWIN1-L1* (*TEA001387*, *WIN1-like 1*) was examined [34]. As shown in Fig. 6E, *CsWIN1-L1* was highly expressed at 24 hai in 'Shuchazao' and at 48 hai in 'Shuixian', whereas its expression levels were relatively low and stable in 'Rougui'.

In general, relative transcript levels of candidate DEGs were low in the control group, but expression was induced to relatively high levels after CLBB1 infection, especially at 48 hai in the cultivar 'Shuixian'. Collectively, data indicated that tea plants activated immune responses with CLBB1 infection. Defense responses were activated at higher levels in 'Shuixian' at 48 hai than at other times, which was consistent with global changes in DEGs and growth of the pathogen.

4 Discussion

Plant pathogens are typically divided into biotrophs and necrotrophs, with biotrophs consuming live plant tissues and necrotrophs killing their host and feeding on dead tissues [35]. In this study, CLBB1 was identified as a new strain of *Ps. vietnamensis*. It is a necrotrophic pathogen isolated from a necrotic lesion of GBD-infected leaves of Wuyi Rock tea *C. sinensis* var. *sinensis* cv. Shuixian in Wuyi Mountain, Fujian Province, a subtropical region of China. Because of the special "Danxia" landform and ecological environment [36], Wuyi Mountain is a unique region suitable for tea production, as well as proliferation of CLBB1. In addition, combined *ITS*, *TUB*, and *TEF* gene regions revealed that *Ps. vietnamensis* strain CLBB1 is different from strains previously identified, which might be related to the unique growing environment of Wuyi Rock tea (Fig. 3; Table 2).

Because infections are limited to tea plant surfaces, particularly those of leaves, early stages of CLBB1 infection in the epidermis were observed microscopically in this study. At 24 hai, CLBB1 conidia germinated, developed appressoria, formed penetration pegs, and penetrated outer epidermal cuticle and cell wall of the host to establish nutritional support for hyphal growth (Fig. 2C). From 48 hai (Figs. 2D–2F), elongated fungal hyphae extended across the leaf surface, leading to plant cell decay. Therefore, 24 and 48 hai were key times for development and spread, respectively, of CLBB1 pathogenicity. Those findings are consistent with growth behaviors of other strains of *Pestalotiopsis* in culture medium [37].

Pestalotiopsis-like species are common phytopathogens that cause GBD in tea plants; however, pathogens of other genera can also cause similar lesions [38,39]. Although other strains were not isolated in this study, it is uncertain whether the lesions observed were caused by a single strain or whether other pathogens, especially endophytes, caused synergistic effects. Germinating spores of the necrotrophs *Botrytis cinerea* and *Alternaria brassicicola* produce cell wall-degrading enzymes and toxins resulting in death and maceration of tissues [40]. Similarly, *Pestalotiopsis* produces phytotoxins and infects undamaged tea leaves [41]. In this study, whether CLBB1 could cause disease on undamaged tea leaves was not determined. On the basis of all data, it was postulated that CLBB1 conidia may germinate on tea leaves alone with wounds or surface breaks and activate enzymes and toxins to ensure infection or with other pathogens that produce enzymes and toxins to assist entry.

To examine the pathogenicity of CLBB1 in Wuyi Rock tea, RNA-seq was performed to analyze DEGs at different times after mechanical introduction of CLBB1 into tea leaves. Comparative analyses of transcriptome data revealed 428, 517, and 369 DEGs in leaves infected with CLBB1 at 24, 48, and 72 hai, respectively (Fig. 4A). The results indicated that the pathogen induced many genes to establish pathogenicity or induce host defense [42,43]. Spores of CLBB1 germinated at 24 hai, and elongated fungal hyphae formed at 48 hai, which developed further at 72 and 96 hai (Figs. 2C–2F). Many DEGs were identified at 24 and 48 hai, suggesting the transition stage from 24 to 48 h was key to germination, growth, and pathogenicity [44,45]. However, the specific genes that control pathogen growth and the associated mechanisms remain unknown.

Plants rely on a complicated innate system to counter invasions of diverse pathogens. Activation of MAPKs (consisting of MAPKKK, MAPKK, and MAPK) is one of the most crucial steps involved in PAMP-triggered immunity (PTI) [46–48]. In this study, CLBB1 activated the MAPK pathway at the transcriptional level, although specific components of the pathway in Wuyi Rock tea remain unclear (Fig. 4B). Expression levels of *CsMAPK3* were highly elevated by CLBB1 in three different cultivars, suggesting the gene had a pivotal role against CLBB1 infection (Fig. 6B). In addition, many chitin-responsive and pathogenesis-related genes were differentially expressed after CLBB1 infection (Figs. 4B, 6A, 6C and 6D). Those results suggested that the MAPK signaling pathway was mediated by chitin recognition following CLBB1 infection and that MAPK cascades further induced extensive expression of *PRs* genes, which constitute the main defense-signaling pathway in PTI. However, the MAPKs and PRs involved in the CLBB1 and Wuyi Rock tea 'Shuixian' interaction still need to be identified and characterized.

Plant hormones also have essential roles in plant immunity, and SA, JA, ET, abscisic acid, auxins, brassinosteroids, gibberellic acid, and cytokinins are activated by pathogen invasion [49–51]. Salicyclic acid signaling pathways are activated as plant defense mechanisms against biotrophic pathogens, and both ET and JA signaling pathways are required to activate plant defense against necrotrophic pathogens [50,51]. In this study, CLBB1 infection activated the plant hormone signal transduction pathway, and many ET-related transcriptional factors were expressed. Results of KEGG analysis indicated that Wuyi Rock tea used the ET-signaling pathway to restrict CLBB1 invasion, a necrotrophic pathogen (Fig. 4B). In addition, ethylene-responsive factor CsERF96 was recently found to have a critical role in the response to GBD [52]. In this study, CsWIN1-L1 was identified as a potential candidate regulating plant defense-related ET pathways in tea (Fig. 6E).

In conclusion, spores on tea leaves began to germinate at 24 hai with CLBB1, and metabolism-related genes were activated to promote pathogen establishment. Tea plants recognized the pathogen via PAMPs, such as chitin, and activated MAPK pathways and expression of pathogenesis-related genes and transcription factors to resist effects of the pathogen. After 48 h, hyphae formed, and tea plants activated additional genes and pathways to restrict the invasion.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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